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ISSR-based DNA profiling of the *Skimmia laureola* population in Pakistan

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This study aimed to analyze the genetic diversity of *Skimmia laureola* population in Pakistan and determine the population pattern of this endangered species. *Skimmia laureola* is a medicinal plant that has been utilized successfully for treatments. The essential oil present in their leaves is used in scenting soap. In Pakistan, it is widespread in Swat, Kashmir hills and Hazara region. Many plants are designated as endangered in IUCN red list. This plant is considered endangered because its population size has shrunk. Population size has shrunk up to 81 percent. The preservation and protection of biodiversity is a current study and conversation hot-button worldwide. Germplasm of *Skimmia* is taken from different regions of Pakistan. Different DNA markers have been used in agriculture, crop genetics and breeding. ISSR is a dominant marker used in genetic study and analysis. Ten ISSR primers were used and different bioinformatics tools were applied to check the diversity and analyze the phylogenetic analysis. The results showed that the total bands or alleles were identified as 1507 and that the number of bands per locus was 91.69. PIC ranges from 0.29435 (ISSR-8) to 0.34830 (ISSR-5). The PCoA showed the clustering of the genotypes with overlapping, indicating possible sisters. UPGMA-based dendrogram studied 15 genotypes into four clusters. The findings of this study contributed to the protection of this threatened plant and helped stop its future extinction.
Keywords: IUCN, Phylogenetic Tree, genetic structure, genetic diversity, dendrogram

INTRODUCTION

Skimmia laureola is an evergreen shrub and small Tree. The aromatic shrub *Skimmia laureola* (*S. laureola*) (DC.) belongs to the Rutaceae family. It is considered endangered species found in China, India, and Pakistan. *Skimmia laureola* flourishes in Pakistan at elevations between 5500 and 10,000 feet. It is widespread in the Murree Hills, Hazara region, Shangla, Kashmir, Upper Swat, and Upper and Lower Dir (Hamayun *et al*., 2007; Ali and Qaiser, 2010). This plant has historically been employed as an insecticide, pesticide, veterinary anthelmintic, and antitussive. (Trombetta *et al*., 2005). It also goes by the names Ner (English), Namer (Pashto), Patar (Nazar Panra), Barru (Kashmiri), Ner (Gujri), Nera (Hindko), and Sheshar (Punjabi) (Shah and Khan, 2006).Molecular markers offer a potent tool for managing and characterizing germplasm appropriately. There are several different classes of molecular markers, like inter-simple sequence repeats (ISSR) and restriction fragment length polymorphism (RFLP) (Zietkiewicz *et al*., 1994). Simple sequence repeats (SSRs), or microsatellites, are tracked down in every eukaryotic genome. Single-locus SSR markers have been produced for various species (Wang, 2002). There is a critical bottleneck in delivering SSR markers since flanking successions should be known to plan 5'- secures for polymerase chain response (PCR) groundworks. The improvement of Inter SSR (ISSR) fingerprinting disposed of the requirement for grouping information (Godwin *et al*., 1997).

Pakistan is experiencing extreme environmental stress due to its rapid urbanization, deforestation, and overuse of natural resources (Jan and Ali, 2009). The amount of Pakistan's flora and fauna populations are falling due to the country's fast-vanishing natural forests, which are disappearing at a pace of 4-6 percent per year (Muhammad *et al*., 2012).

Simple sequence repeats (SSR) markers are frequently thought of as the best molecular markers because they have several significant advantages over AFLP, SRAP, ISSR, and RAPD markers, including high reproducibility, wide distribution, high transferability, high-reliability co-dominance, and high polymorphism in addition to being relatively inexpensive. In the current study, the genetic

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diversity of the *Skimmia laureola* population in Pakistan was examined using ten ISSR primers. Using the PopGen32 computer program version 1.32, Nei's similarity index, which depicts genetic distance and identity, was calculated. Additionally, PAST software was used to construct phylogenetic trees using UPGMA algorithms and Euclidean measurements.

This research was used to analyze the genetic diversity of *Skimmia laureola* population in Pakistan.

MATERIALS AND METHODS

***Plants Sampling*:** The endangered species, *Skimmia laureola*, plant leaves were collected from different regions of Pakistan. Plant material was collected from different areas of Murree, Hazara and Kashmir. Fresh, young, juvenile leaves of *Skimmia laureola* were collected to experiment.

***DNA Isolation and PCR analysis*:** DNA was isolated using the method Ijaz *et al*. described (2018). The analysis was performed on 96-well thermal cycler peq STAR. Ten ISSR markers were used in PCR. The PCR amplification products run on 2% agarose gel were visualized on the gel documentation system.

***Data collection and analysis*:** Band counting was scored as "1" (for presence) and "0" (for absence). DARwin6 v. 6.0, PAST v. 3.16 and PopGen 32 v. 1.32 were employed for genetic diversity analysis. For population structure, the software STRUCTURE v. 2.3.4 was used.

RESULTS

The banding pattern of 10 ISSR markers was observed after resolving of PCR product on agarose gel and UV visualization (Fig.1). The 10 ISSR markers produced 1507 bands/alleles and the observed polymorphic bands/alleles were 986. One hundred sixty-four loci were amplified using 10 ISSR primers, ranging from 12 (ISSR-18) to 21 (ISSR-8), all of which seem polymorphic. The total bands or alleles were identified as 1507, which ranged from 98 (ISSR-18) to 230 (ISSR-8), with an average value of 150.7 bands per primer. The number of bands per locus was 91.69 on average, ranging from 8.17 (ISSR-18) to 13.26 (P-46). The total polymorphic bands were observed as 971, ranging from 82 (ISSR-18) to 123 (ISSR-4). The polymorphic percentage ranges from 36.91 (ISSR-8) to 88.18 (ISSR-5), averaging 68.28%. To check the marker diversity, PIC ranges from 0.29435 (ISSR-8) to 0.34830 (ISSR-5) with a mean of 0.32529, expected gene diversity ranges from 0.33352 (ISSR-18) to 0.44215 (ISSR-19) with mean of 0.38724 and major allelic frequency ranges from 0.616666 (P-46) to 0.730158 (ISSR-8) with mean of 0.661422.

***Population genetic analysis*:** UPGMA and Unweighted Neighbor-joining (NJ) were used for 15 *Skimmia laureola* genotypes for population genetic analysis. UPGMA-based dendrogram (phylogenetic Tree) was generated in PAST v.3.16. In this dendrogram, 15 studied genotypes were grouped into four major clusters (I, II, III and IV) (Fig 2). Cluster I comprised of MRP1, HZP4, HZP5, HZP1 and HZP3, in which MRP1 rooted Hazara genotypes (HZP4 and HZP5). HZP1 and HZP3 seemed closely related, while HZP4 and HZP5 were sister genotypes. Cluster II comprised KSP2 and MRP2, which seemed to be sister genotypes and HZP2 rooted them. Cluster III comprised MRP5, KSP1, MRP4 and MRP3. KSP1 and MRP4 seemed to be sister genotypes, while MRP5 rooted the three genotypes. In this dendrogram pattern, Kashmir genotypes, namely KSP4 and KSP5, formed a separate cluster (IV) diverse from all other groups.

Estimated subpopulations by delta K (ΔK) values obtained through STRUCTURE HARVESTER was highest at K=3, depicting *Skimmia laureola* genotypes three subpopulations. These results depicted the strong relationship between the *Skimmia laureola* of the region Murree and Kashmir. This sub-population grouping of 15 *Skimmia laureola* genotypes indicates that these genotypes share three gene pools and mixed ancestry.

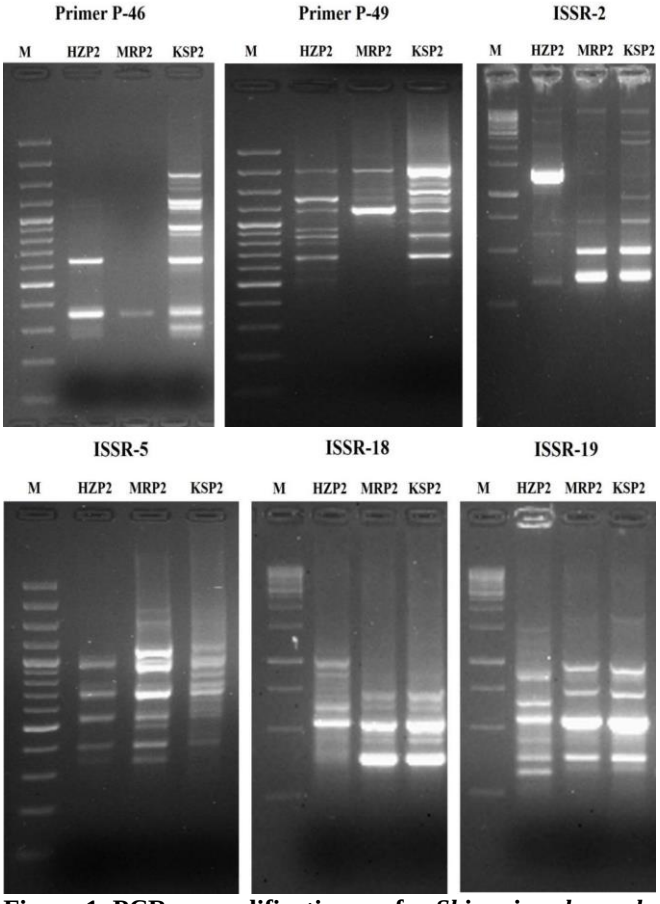

Figure 1. PCR amplification of *Skimmia laureola* population using 10 ISSR primers. *M = 1kb DNA ladder.

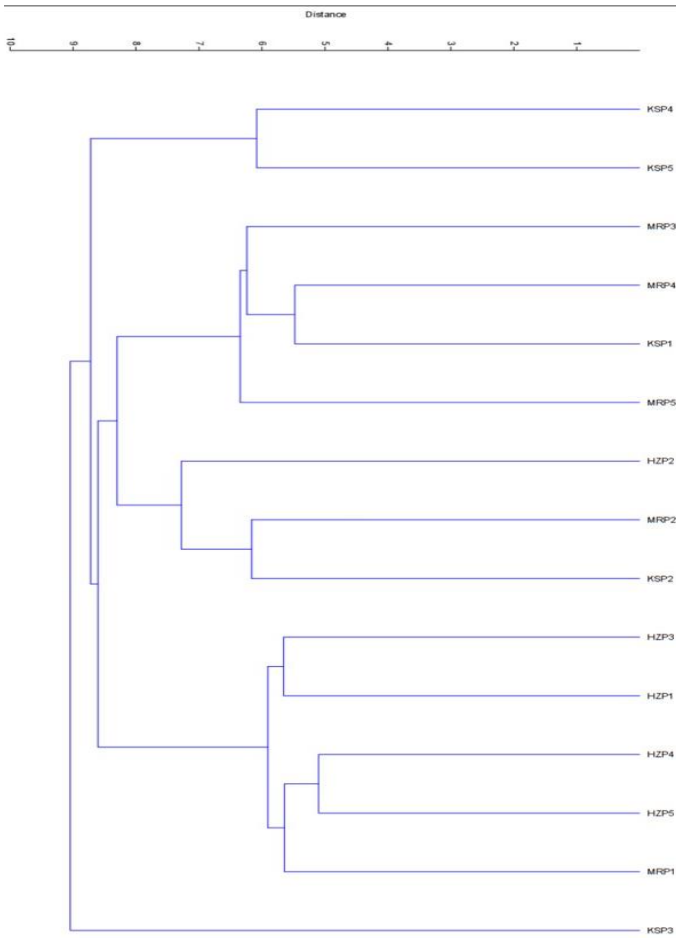

Figure 2. A UPGMA dendrogram based on Nei's genetic distance, showing the clustering of 15 *Skimmia laureola* genotypes collected across Hazara, Murree and Kashmir.

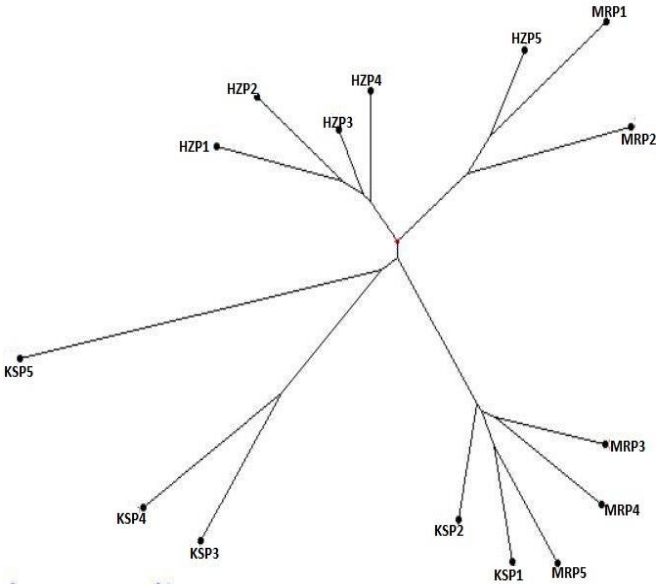

Figure 3. Un-weighted group method-based Neighbor-Joining Tree of 15 *Skimmia laureola* genotypes using DARwin6

The similarity value ranged from 0.1738 (17%) to 0.8160 (81%) (Table 1). The genotypes found as a sister in cluster analysis have a similarity index of HZP1 and HZP3 at 80%, HZP4 and HZP5 at 81%, KSP2 and MRP2 at 76%, KSP1 and MRP4 at 50%, and for KSP4 and KSP5 as 77%. Thus, the similarity matrix result also confirmed the phylogenetic tree analysis results.

To comprehend the spatial representation of genetic distance among the *Skimmia laureola* population, Principal Coordinate Analysis was carried out. For this analysis, the DARwin6 computer program was also used to evaluate the

Table 1. Marker informativeness of 10 ISSR primer

Sr#	Primers	Amplified loci	Total number of bands	No. of bands per locus	No. of polymorphic bands	Polymorphism (%)	Expected Gene diversity	Major allele frequency	PIC
1	P-46	15	199	13.26	88	44.22	0.34778	0.61667	0.31548
2	P-49	16	133	8.31	97	72.93	0.41333	0.66250	0.32201
3	ISSR-2	14	127	9.07	83	65.35	0.43302	0.63809	0.31866
4	ISSR-4	18	141	7.83	123	87.23	0.38722	0.62999	0.30139
5	ISSR-5	17	127	7.47	112	88.18	0.42673	0.67059	0.34830
6	ISSR-6	18	131	7.28	109	83.20	0.40555	0.68333	0.33322
7	ISSR-8	21	230	10.95	85	36.91	0.33608	0.73016	0.29435
8	ISSR-9	18	185	10.28	103	55.67	0.34112	0.66259	0.32166
9	ISSR-18	12	98	8.17	82	83.67	0.33352	0.69886	0.33299
10	ISSR-19	15	136	9.07	89	65.44	0.44215	0.62145	0.33485
	Total	164	1507	91.69	971	-------	0.38724	0.66142	0.32529
	Mean	16.4	150.7	------	97.1	68.28			

*Polymorphic information contents. **Expected gene diversity

constancy of genetic differentiation among 15 genotypes. According to PCoA, genetic variation in 15 genotypes was spatially represented as a group viz., HZP1, HZP2, HZP3, HZP4 and HZP5 were present as one cluster, while MRP3, KSP2, KSP3 and KSP4 were present together as a mixed group. However, KSP1, KSP5, MRP1, MRP2, MRP4 and MRP5 seems to be scattered. The PCoA further showed the cluster of the overlapping genotypes, indicating possible sisters. This is important in the identification of genetically similar and genetically distinct genotypes.

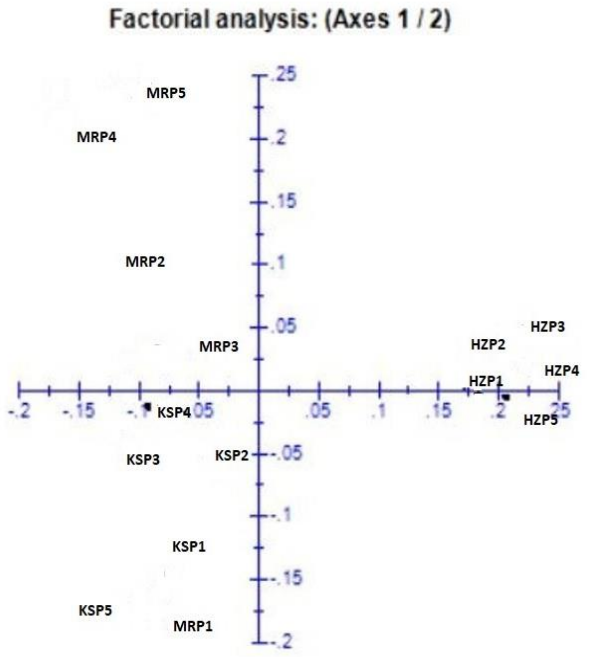

Figure 4. Spatial representation of selected 15 *Skimmia* laureola genotypes using PCoA analysis in DARwin6

***Population genetic structure*:** It suggested the *Skimmia* population into subpopulations (K) and estimated subpopulations by ΔK value. The recorded highest ΔK value was K = 3, which delineates the population *Skimmia* into three subgroups (Fig. 7) (Table 2) and shows the sharing of three gene pools (Fig.7).

Table 2. The Evanno table obtained from Structure Harvester displayed ΔK value with maximum K-clustering (sub-population grouping)

K	Reps	Mean LnP(K)	Stdev LnP(K)	LN'(K)	Ln''(K)	Delta K
2	3	-1320.4	01.60	-	-	-
3	3	-1260.6	23.77	59.80	33.30	1.4007
4	3	-1234.1	27.39	26.50	23.20	0.8470
5	3	-1184.4	10.07	49.70	-	-

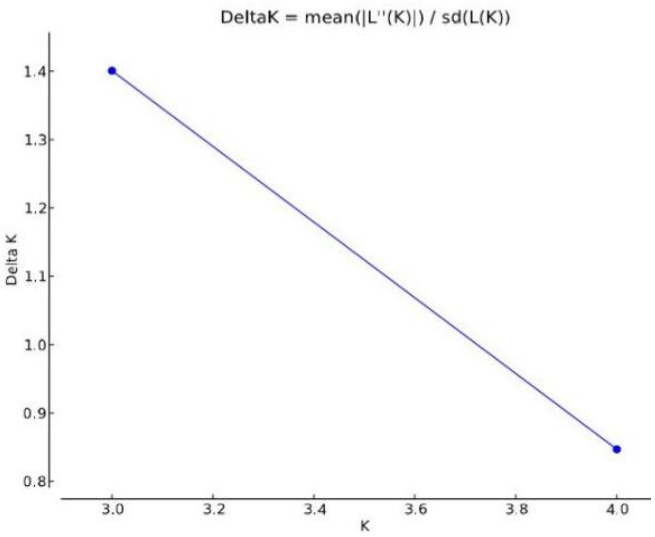

Figure 6. The graphical representation of Evanno table output showing maximum delta value at K=3

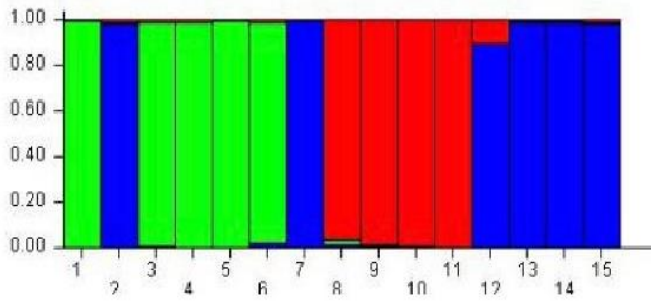

Figure 7. STRUCTURE analysis represents the population; the number on the horizontal axis shows the individuals belong to Skimmia populations and the vertical axis shows the membership coefficient to subpopulations

DISCUSSION

Medicinal plants are a crucial supply of medications used to address various health issues. Pakistan is home to around 6000 distinct types of wild plants, 400-600 of which are regarded to have medicinal value. The endangered plant species of *Skimmia laureola* from different regions of Pakistan are the subject of this study. This research aimed to analyze the genetic diversity of *Skimmia laureola* population in Pakistan.

In the present study, The 10 ISSR markers produced 1507 bands/alleles and the observed polymorphic bands/alleles were 986. A total of 164 loci were amplified using 10 ISSR primers, ranging from 12 (ISSR-18) to 21 (ISSR-8), all of which seem to be polymorphic. The total bands or alleles were identified as 1507, which ranged from 98 (ISSR-18) to 230 (ISSR-8), with an average value of 150.7 bands per primer.

The genotypes found as a sister in cluster analysis have a similarity index of HZP1 and HZP3 at 80%, HZP4 and HZP5 at 81%, KSP2 and MRP2 at 76%, KSP1 and MRP4 at 50%, and for KSP4 and KSP5 as 77%.
Principle Coordinate Analysis (PCoA) is a widely applied tool in genetic diversity findings representing the spatial representation of studied genotypes. The component analysis was performed using a two-dimensional plot with DARwin6. Genetic variation percentages were also computed for 2 to 5 coords. The delta K value revealed the sharing of three genetic pools for the skimmia population.

***Conclusion*:** The phylogenetic trees showed a divergence (%) from the studied endangered plants. Hence, the dendrogram-clustering pattern supported the result of the dendrogram generated using the UPGMA method with little alterations due to different algorithms used in different software.

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***Ethical statement*:** This article does not contain any studies with human participants or animal performed by any of the authors.
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